Effects of Zn^{2+} and Cu^{2+} on loach ovaries and ova development

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Abstract: This study compared the accumulation of Zn^{2+} and Cu^{2+} in the ovaries and ova of loaches under different concentrations of Zn^{2+} (1.00, 2.50 and 5.00 mg/L respectively) and Cu^{2+} (0.10, 0.25 and 0.50 mg/L respectively). The results showed that both Zn^{2+} and Cu^{2+} accumulated in the ovaries, and that the relationship between accumulation and time was linear over 20 days of exposure. The accumulation of the metals in ovaries was closely related to the concentration of exposure in the solutions (P<0.05), and was obviously affected by the time and doses. However, the Cu^{2+} concentration was significantly higher than Zn^{2+} (P<0.05). The development level of ova in the ovaries also correlated with the concentration and exposure period in the Zn^{2+} and Cu^{2+} solutions.

Keywords: *Misgurnus anguillicaudatus*; Ovary; Ovum; Zn; Cu; Accumulation

The problem of heavy metal pollution in aquatic environments has become an increasingly serious concern over the past decades in China (Wang et al, 2008). The resulting pollution that accompanies industrialization has severely challenged the survival of aquatic animals due to strong toxicity and the bioaccumulation of heavy metals. Of these pollutants, metal ions accumulate not only in the skin, muscles, liver, and kidneys (Al-Weher S et al, 2008; Dutta T et al, 2001; Has S et al, 2008; Migliarini et al, 2005), but also in the gonads, which poses a serious threat to the reproductive success of many aquatic species and by extension the overall population of those animals (Abou El-Naga et al, 2005; da Cruz et al, 2007; Tang et al, 2010). Accordingly, to maintain biodiversity and protect the breeding system of aquatic fauna, it is necessary to conduct studies on the accumulation of heavy metals in a fish's reproductive organs and the resulting stress on their ova to thereby establish correlations between metal accumulation and ion concentration.

Zn^{2+} and Cu^{2+} are commonly found in bodies of water polluted by heavy metals. Neither biodegrades easily and these metals are amplified via bioaccumulation as they pass through food chain. To explore how heavy metal accumulation strains the development of the ovaries and ova, in this study we used Loaches (*Misgurnus anguillicaudatus*), a fish species known to tolerate pollution well (Gao et al, 2003; Wang et al, 2003) as a model. By doing so, we hope to find results that will prove useful in gauging the impact assessment of aquatic organisms, diagnostics of environmental pollution, and biodiversity conservation.

MATERIALS AND METHODS

Animal specimens and reagent materials

Two year old loaches (n=220) were purchased from a local farmer's market for use as a testing species. The overall mean length of the fish was 13.2±3.5 cm and the mean body weight 37±0.48 g.

Concentrated solutions (1 000 mg/L) of $ZnSO_4$ (AR) and $CuCl_2$ (GR) were first prepared before being diluted into the corresponding concentrations as needed throughout the course of the experiment. HNO_3 (AR) and $HClO_4$ (AR) were mixed at a ratio of 4:1 before use.

Instruments and equipments

The following equipment were used over the duration

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of this study: an H-6800 Aerating Pump (China); an IRIS Intrepid ER/S-model ICP atomic emission spectrometer (Thermo Elemental Co., USA); a CKX41 inverted fluorescent microscope (HQ2592×1944) (Olympus Co., Japan); an LSP far-infrared cooking stove (China); a KD (freeze) slicing machine (China); and a DGG-9070A drying oven with an electrical thermostatic wind drum (China).

Experimental design

The experiment was carried out indoors. During testing, the water quality parameters were maintained consistently: pH 6.3−6.5, DO 5.1−6.2 mg/L; temperature at 8−12°C (measured daily); average total water hardness at 2.67 mmol/L; and average alkalinity at 2.6 mmol/L.

Polyethylene plastic aquariums (40 cm×30 cm×45 cm) were used as exposure devices, and 20 L of tap water, in which no Zn^{2+} and Cu^{2+} was detected, was added to each tank and then aerated for more than 2 days prior to being used. The loaches accepted for testing were visually inspected to be disease-free, injury-free and active, with an overall mortality rate less than 5% during the testing period. The fish were acclimated to lab conditions for 5 days before the experiment began. Over the course of testing, there was no water exchange in the plastic aquariums and the fish were not fed. An aerating pump was used to keep dissolved oxygen above 5 mg/L.

In total, 220 similarly sized loaches were randomly placed into four testing groups. The control group was placed in water with no heavy metals present, while the other three were given different concentrations of Zn^{2+} and Cu^{2+} similar to actual ion concentrations in the local water bodies based on the Chinese Standard of Water Quality for Fisheries (GB11607-1989): Zn^{2+} concentrations of 1.00, 2.50 and 5.00 mg/L paired with Cu^{2+} concentrations of 0.10, 0.25 and 0.50 mg/L. The fish were held in each aquarium and three parallels for each concentration over the course of the 20-day experiment. Each individual was weighed, boiled, and dried prior to analysis, during which ovary slices from different groups and at different times (before testing, 5-day, 10-day and 20-day) were cut and observed.

Statistical analysis

The processing method of statistics for the testing results follows the method described by Nan Xu-Yang (Nan, 2009). All data was analyzed using SPSS 11.0 data processing system (SPSS Inc., Chicago, USA) for analysis of variance. The values for individual experiments were collected to calculate the mean value and the standard deviation/error to make comparisons. The statistical significance of the difference between means was determined using one-way ANOVA, with $P<0.05$ being statistically significant.

RESULTS

Accumulating rule of Zn^{2+} and Cu^{2+} in ovaries

Before exposure to the metals, Zn^{2+} and Cu^{2+}, all loaches were tested to ensure that no metals were present in the ovaries. By 10 days of exposure, the concentrations of Zn^{2+} and Cu^{2+} in loach ovaries across all groups being kept in water with varying concentrations of heavy metals had increased. On day 20, the concentration of both ions showed an increasing trend, but between day 10 and day 20 the concentrations of Zn^{2+} and Cu^{2+} in the ovaries seemed to decline significantly when compared to ion concentrations of the groups when they had been exposed for less than 10 days ($P<0.05$).

The cumulative capacity of Zn^{2+} and Cu^{2+} in the ova was positively related to concentration of the metals in aqueous solution ($P<0.05$). The accumulation of Zn^{2+} and Cu^{2+} in the ovaries presented as a function of exposure time and dose effects are shown in Table 1. In the control group, no Zn^{2+} and Cu^{2+} were detected.

Table 2 shows the regression equations and correlation coefficients for ovaries, indicating a positive relationship between Zn^{2+} and Cu^{2+} accumulation and exposure time. Relationships between accumulation of Zn^{2+} and Cu^{2+} in the ovaries and exposure time are shown in Figures 1 and 2.

Table 1 Accumulation of Zn^{2+} and Cu^{2+} in ovary at different time intervals (n=200)

Time (d)	Treatment	Zn^{2+} / Cu^{2+} in solution (mg/L)	Zn^{2+} (μg/g; dry wt)	Cu^{2+} (μg/g; dry wt)
5	control	—	—	—
	group 1	1.00 / 0.10	1.3±0.4^{g}	3.4±0.2^{g}
	group 2	2.50 / 0.25	2.5±0.4^{fg}	5.6±0.8^{g}
	group 3	5.00 / 0.50	3.6±0.7^{def}	8.9±0.9^{g}
10	control	—	—	—
	group 1	1.00 / 0.10	2.3±0.7^{ef}	54.3±2.5^{f}
	group 2	2.50 / 0.25	5.1±0.2^{d}	81.5±4.7^{e}
	group 3	5.00 / 0.50	26.4±1.7^{b}	145.33±5.6^{b}
20	control	—	—	—
	group 1	1.00 / 0.10	4.3±0.3^{de}	117.1±6.7^{d}
	group 2	2.50 / 0.25	7.6±0.4^{c}	137.2±11.0^{c}
	group 3	5.00 / 0.50	39.1±3.2^{a}	329.3±18.4^{a}

Different superscripts show significant difference（P < 0.05）, while the same superscript shows no significance（P>0.05）between groups.

Table 2 Regression equations and coefficients of accumulation for Zn^{2+} and Cu^{2+} with exposure time in ovary

Treatment	Heavy metal	Regression equation	Correlation coefficient (R^2)
group 1	Zn	y=0.185x+0.7408	0.7834
	Cu	Y=9.3235x–30.158	0.9543
group 2	Zn	y=0.3254x+1.2608	0.9355
	Cu	Y=9.3654x–15.307	0.8286
group 3	Zn	y=2.2072x–2.7333	0.8648
	Cu	y=24.754x–87.721	0.9716

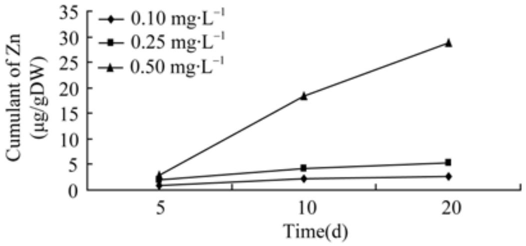

Figure 1 Relationship of accumulation of zinc and time in ovary

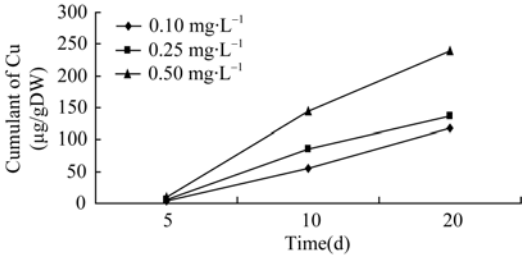

Figure 2 Relationship of accumulation of copper and time in ovary

Stress on ovary development from Zn^{2+} and Cu^{2+}

The ovaries and ova in the controls, in which the metals were not detected, developed normally. Zn^{2+} and Cu^{2+} accumulated rapidly in the ovaries of all treatment groups exposed to mixtures of Zn^{2+} and Cu^{2+}. The fish showed on the atrophy and cytoplasmic leakage in the ova, suggesting histological damage in the ovaries by day 10 of exposure. By day 20, the ova showed symptoms of severe degeneration, the mutual bonding of cells and severe atrophy (Figure 3).

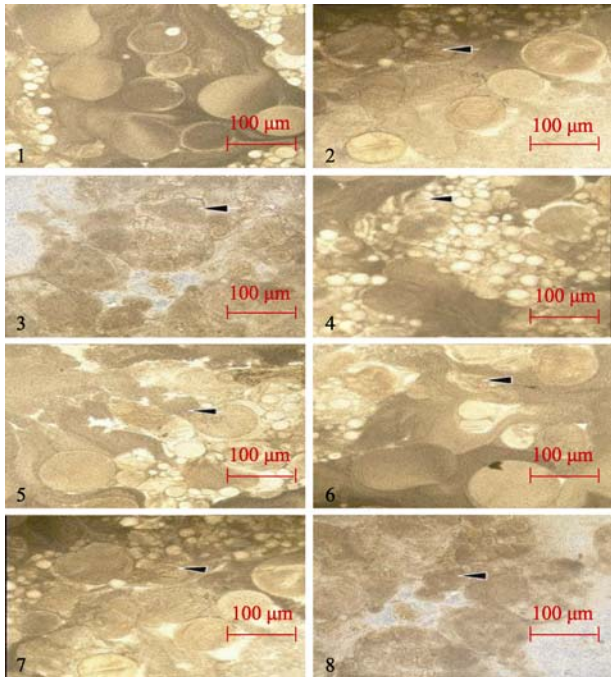

Figure 3 Effects of joint attack of Zn^{2+} and Cu^{2+} on ovaries and ova

a: Normal development of ovaries and ova in the control; b:5 d ovaries and ova, the arrow shows the cell degenerated in group one; c:10 d ovaries and ova, the arrow shows the cell dehydrated in group one; d:10 d ovaries and ova, the arrow shows the cell atrophic and dehydrated in group two; e:10 d ovaries and ova, the arrow shows the cell atrophic and shriveled in group three; f:20 d ovaries and ova, the arrow shows the cell atrophic and mutual bonding in group one; g:20 d ovaries and ova, the arrow shows the cell atrophic and mutual bonding in group two; h:20 d ovaries and ova, the arrow shows the cell atrophic and mutual bonding in group three.

DISCUSSION

As trace elements of an organism, Zn^{2+} and Cu^{2+} are integral parts of cellular structure and enzyme compounds. Zn^{2+} is one of the important growth factors and plays a vital role in growth, development and appetite, etc. for fishes and other aquatic organisms (Al-Weher S, 2008; Ebrahimi M, 2007). However, studies have shown that higher doses and longer exposure are not beneficial, and have toxic effects on aquatic organisms (Zhou et al, 2002). As for Cu^{2+}, it is also a type of nutritive trace element necessary for fish growth (Gao et al, 2003), and it performs important functions for the physiological activities in fishes. The physiological metabolism will be negatively influenced if either Zn^{2+} or Cu^{2+} is lacking, but overdosing brings adverse impacts, especially in the case of Cu^{2+}. In general, the harmfulness of Zn^{2+} is relatively small as compared with Cu^{2+}, which is highly toxic to aquatic organisms in stronger dosages (Wang et al, 2006).

As mentioned above, the amount of Zn^{2+} and Cu^{2+} accumulated in the ovaries was related to the concentration of Zn^{2+} and Cu^{2+} in the aquatic environment and the duration of exposure to the metals. The cumulative amount of heavy metal in the ovaries rises with the concentration of Zn^{2+} and Cu^{2+} to a certain level alongside the exposure period, demonstrating the significant effect of time and doses. However, the accumulation rate tended to decline when exposure time exceeded 10 days. It could be that when organisms are over-exposed to heavy metals such as Zn^{2+} and Cu^{2+}, the

metals activate the transcription of metallothionein genes in the organs, resulting in more genes being expressed. Consequently, these expressed genes combine the metals that have entered the cells with those new synthetic proteins (Allen, 1995). Alternatively the cells over-exposed to the heavy metals are in a state of "saturation" and thus can no longer absorb more heavy metals. As exposure continues, the level of heavy metal accumulation declined in the ova, which was more obvious in the cases of different concentrations of Zn^{2+} (Figure 1). As for the accumulation of Cu^{2+} under different concentrations, the higher the concentration of Cu^{2+}, the slower the accumulation tended to be. It seemed that the cumulated amount of Cu^{2+} increased in the ova between 10 to 20 days, when the concentration was low, i.e., 0.1 mg/L (Figure 2).

Ultimately, these results indicate that the ability of the ovaries to detoxify decreased and the ova were damaged as a result of 20 day's exposure to heavy metal at higher concentrations. Long term exposure to heavy metals will likely then further damage the overall function of ovaries (Figure 3), which is in agreement with previous studies regarding organs in aquatic organisms being damaged by heavy metals (Wang et al, 2003; Zhang et al, 2009; Zhou et al, 2010).

Our results also showed that when both Zn^{2+} and Cu^{2+} were at a low concentration (1.00 and 0.10 mg/L, respectively), the physiological activities and ovarian development of loaches were not affected by Zn^{2+} and Cu^{2+} within 20 days of exposure, though future studies are needed to determine the effects of long-term exposure at similar concentrations. As the concentration of the metals and/or exposure time increased the function of ovarian development and the metabolism of ova suffered serious damage. Studies of single metal exposure showed that female sword fish *(Xiphophorus Helleri)* on average laid only 17 eggs when exposed to Cu^{2+} (0.12 mg/L) for 140 days, compared with the 228 eggs laid in the control group (James et al, 2003). But the effect of single metal exposure can be very different from multi-metal exposure on aquatic organisms in terms of toxicity accumulation. In a natural environment, a synergic or antagonistic action may occur as different heavy metals usually co-exist (Zhang et al, 2008). In addition, factors such as water temperature, forms of heavy metals, existence of other chemical ions and changes in other environmental variables may affect the heavy metal accumulation and cause harm to aquatic organisms (Li et al, 2002; Tang et al, 2010). Further studies, therefore, are needed to address these issues.

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